15:58:29 OCA PAD AMENDMENT - PROJECT HEADER INFORMATION 08/09/93

Active

Project #: G-32-629 Cost share #: Rev #: 7
Center # : 10/24-6-R7411-0A0 Center shr #: OCA file #:
Work type : RES
Contract#: OCE-9115860 Mod #: AMENDMENT 002 Document : GRANT
Prime #: Contract entity: GTRC

Subprojects ? : Y CFDA: 47.050
Main project #: PE #: N/A

Project unit: BIOLOGY Unit code: 02.010.134
Project director(s):
SNELL T W BIOLOGY (404)894-3700

Sponsor/division names: NATL SCIENCE FOUNDATION / GENERAL
Sponsor/division codes: 107 / 000

Award period: 920101 to 940630 (performance) 940930 (reports)

Sponsor amount	New this change	Total to date
Contract value	8,891.00	108,887.00
Funded	8,891.00	108,887.00
Cost sharing amount		0.00

Does subcontracting plan apply ?: N

Title: CHEMICAL COMMUNICATION AMONG MARINE ZOOPLANKTON

PROJECT ADMINISTRATION DATA

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Security class (U,C,S,TS) : U ONR resident rep. is ACO (Y/N): N
Defense priority rating : N/A NSF supplemental sheet
Equipment title vests with: Sponsor GIT X

Administrative comments -
AMENDMENT 2 ADDS $8,891 TO PROJECT. FUNDS WILL BE BUDGETED IN SUBPROJECT G-31-608 AS REQUESTED BY LETTER DATED 6-14-93.

15:58:29 SUBPROJECTS OF MAIN PROJECT G-32-629 08/09/93

Project number	Spon/Div	Project Director Total Contract	Project Unit Total Funded
G-32-634	107/000	SNELL T W 50,000.00	BIOLOGY 50,000.00
G-31-608	107/000	FOX J H 8,891.00	DEAN COS 8,891.00

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 10/26/94

Project No. G-32-629 Center No. 10/24-6-R7411-0A0

Project Director SNELL T W School/Lab BIOLOGY

Sponsor NATL SCIENCE FOUNDATION/GENERAL

Contract/Grant No. OCE-9115860 Contract Entity GTRC

Prime Contract No. _____

Title CHEMICAL COMMUNICATION AMONG MARINE ZOOPLANKTON

Effective Completion Date 940630 (Performance) 940930 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	N	
Final Report of Inventions and/or Subcontracts	N	
Government Property Inventory & Related Certificate	N	
Classified Material Certificate	N	
Release and Assignment	N	
Other _____	N	

Comments
LETTER OF CREDIT APPLIES. 98A SATISFIES PATENT REQUIREMENT.

Subproject Under Main Project No. _____

Continues Project No. _____

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Managment	Y
Research Security Services	N
Reports Coordinator (OCA)	Y
GTRC	Y
Project File	Y
Other _____	N
_____	N

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT (SUBPROJECTS)

Closeout Notice Date 10/26/94

Project No. G-32-629 Center No. 10/24-6-R7411-0A0_

Project Director SNELL T W_____ School/Lab BIOLOGY_____

Sponsor NATL SCIENCE FOUNDATION/GENERAL_____

Project # G-32-634 PD SNELL T W Unit 02.010.134 T
GRANT # OCE-9115860 MOD# BR DTD 931027 BIOLOGY *
Ctr # 10/24-6-R7411-0A1 Main proj # G-32-629 OCA CO JLB
Sponsor-NATL SCIENCE FOUNDAT /GENERAL 107/000
CHEMICAL COMMUNICATI
Start 920101 End 940630 Funded 50,000.00 Contract 50,000.00

Project # G-39-608 PD FOX J H Unit 02.010.146 T
GRANT # OCE-9115860 MOD# ADMIN. CEISMC *
Ctr # 10/24-6-R7411-0A3 Main proj # G-32-629 OCA CO JLB
Sponsor-NATL SCIENCE FOUNDAT /GENERAL 107/000
CHEMICAL COMMUNICATI
Start 920101 End 940630 Funded 8,891.00 Contract 8,891.00

LEGEND
1. * indicates the project is a subproject.
2. I indicates the project is active and being updated.
3. A indicates the project is currently active.
4. T indicates the project has been terminated.
5. R indicates a terminated project that is being modified.

G32-629
1

NSF Annual Report 1992
OCE-9115860

1) Publication List

Snell, T.W. and P.D. Morris. 1992. Sexual communication in copepods and rotifers. Hydrobiologia, in press.

Snell, T.W., P.D. Morris, & G.A. Cecchine. 1992. Localization of the mate recognition pheromone in the marine rotifer *Brachionus plicatilis*. J. Exp. Mar. Biol. Ecol., in press.

2) Scientific Collaborators

Research Assistants 1992

NAME	CURRENT STATUS
Traci Battle	senior undergraduate
Michael Blakeney	senior undergraduate
Marcus Vogt	senior undergraduate
Meeta Jarim-Singh	first year Ph.D. student
Roberto Rico-Martinez	first year Ph.D. Student

3) Technical Description of Project and Results

A) Isolation of the Mate Recognition Pheromone from Marine Rotifers

We have developed some new approaches to the isolation and characterization of the glycoprotein responsible for mate recognition in the marine rotifer *Brachionus plicatilis*. Sequential affinity chromatography using the lectins *Lens culinaris* and *Tetragonolobus purpureas* has proven effective. The first lectin has affinity for mannose/glucose units and the second for fucose. Eluents from the Tetra affinity column contained a 29 kD glycoprotein that has all the characteristics of the rotifer mate recognition pheromone (MRP). We are now using molecular ultrafiltration to remove contaminating proteins in the hopes of purifying the MRP to a sufficient degree so that monospecific polyclonal antibodies can be prepared against it. These antibodies will simplify the purification of the MRP from several closely related *B. plicatilis* strains and provide us with probes for comparing variation among populations.

B) Development of a New Bioassay for Rotifer MRP Activity

We developed a new strategy for assaying MRP activity this year. The test is based on biotintylation of the MRP and its binding to the male receptor. A procedure for attaching biotin to the MRP was developed and optimized. Males are then incubated with the MRP-biotin complex for 15 min to allow the MRP to bind to its receptor in the male corona. After all unbound MRP is washed away, avidin labelled with fluorescein is reacted with the MRP-biotin and binding sites can be visualized as epifluorescence. The extent of binding is quantified with an image analysis system. The advantage of this technique is that it uses male receptors to detect the MRP and this is the most biologically relevant reaction in mating..

C) Surface Glycoproteins Potentially Involved in Mating in Marine Copepods

We have examined the binding of several lectins to the surface of three species of marine copepods. The hypothesis in these experiments is that surface glycoproteins on the urosome of female copepods are a signal to males in mate recognition. From mating behavior analyses it is known that calanoid males contact females across the terminus of their caudal furca with the male's right antennae. If contact is not made at this location, mating is aborted. This behavior pointed to a signal emanating from the caudal furca that requires contact to be received. These are precisely the conditions that suggest the presence of a surface glycoprotein. Our survey of the binding of ten lectins to the urosomes of female *Labidocera aestiva*, *Acartia tonsa*, and *Centropages hamatus* clearly showed the presence of glycoproteins in the correct regions to serve as mating signals. We are in the process of interpreting the lectin binding patterns as clues to the structure of the glycoproteins. This coming year we will expand the number of species investigated to include marine harpacticoid and cyclopoid copepods.

In press: J. Exp. Mar. Biol. + Ecol.

Localization of the mate recognition pheromone in *Brachionus plicatilis* O.F. Muller (Rotifera) by fluorescent labeling with lectins

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Key words: rotifers, pheromone, lectins, glycoproteins, mate recognition

Running Head: localization of rotifer mate recognition pheromone

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Abstract

Mate recognition in the rotifer *Brachionus plicatilis*, O.F. Muller is based upon male contact chemoreception of a glycoprotein pheromone on the body surface of females. The highest densities of this glycoprotein were found on the corona of females as determined by fluorescence microscopy. A times series experiment demonstrated that binding sites on the corona fluoresced before any other structure and did so most intensely. The lorica margin of females fluoresced weakly after 20 minutes of exposure, suggesting that mate recognition pheromone (MRP) is present, but in low density. In males, the corona also was the most intensely labeled structure on the body surface. Pre-treatment of rotifers with the lectins Concanavalin A, *Lens culinaris*,*Vicia faba*, and *Pisum sativum* blocked subsequent labeling with ConA-FITC. Exposure to other lectins did not block ConA-FITC labeling. Quantitation of fluorescence intensity using image analysis demonstrated that the contrast between a glycoprotein signal and background ranged from 2 to 19.5. *In situ* degradation of the MRP by the glycohydrolase N-glycanase reduced lectin binding and the intensity of fluorescent labeling. Since N-glycanase degylcosylation of glycoproteins on the body surface of live females sharply reduced their ability to elicit male mating reactions, it is clear that the same oligosaccharides critical for lectin binding also are essential for mate recognition. These lectin probes have enabled us to visualize the distribution of the MRP on the body surface of females and provided insight into the copulatory behavior of males.

Introduction

Mate recognition in the marine rotifer *Brachionus plicatilis* O.F. Muller occurs as a result of male contact chemoreception of a precise signal on the body surface conspecific females (Gilbert, 1963; Snell & Hawkinson, 1983). This signal is the basis of mating discrimination among temporally and geographically isolated populations and the extent of divergence in signal recognition is related to the degree of allopatry (Snell & Hawkinson, 1983). The role of mate recognition in the establishment and maintenance of species boundaries in rotifers has been described by Snell (1989). The role of contact chemoreception as a general solution to the problem of sexual communication in zooplankton was discussed by Snell & Morris (1992).

These studies stimulated a series of experiments to examine the biochemical mechanisms of mate recognition in rotifers and how they promote the origin of discontinuities in species' gene pools. Snell *et al* (1988) showed that the female signal is a heat labile glycoprotein susceptible to protease and glycohydrolase degradation. They further suggested that the carbohydrate moiety of the molecule is essential for eliciting mating responses from males. Lectins were used by Snell & Nacionales (1990) to block male recognition of females, greatly altering mating behavior. Lectins are carbohydrate-binding proteins of non-immune origin that selectively bind to oligosaccharides of glycoproteins (Goldstein & Poretz, 1986). The specificity of lectins for particular oligosaccharides is similar to that of enzymes for their substrates. When female *B. plicatilis* were treated with the lectins ConA, lentil, pea, and fava, the probability of male copulation was reduced 75-95% from control levels. These lectins all have affinity for terminal -D-glucosyl and -D-mannosyl residues (Goldstein & Poretz, 1986). Exposure to several other lectins with different affinities had no effect on mate recognition. Snell & Nacionales (1990) also

showed that the zwitterionic detergent CHAPS as well as chelating agents EDTA and EGTA removed the signal glycoprotein from females, suggesting that it is a loosely bound surface glycoprotein.

The carbohydrate dependence of mate recognition can be demonstrated by enzymatic modification of cell surface glycoproteins (Sharon & Lis, 1989). When live female *B. plicatilis* were treated with the glycohydrolase N-glycanase which specifically cleaves oligosaccharides N-linked to asparagine residues, the probability of male copulation was reduced 65% (Snell & Nacionales, 1990). These observations, along with the results described above, clearly demonstrate the critical role of oligosaccharides in rotifer mate recognition as well as the ability of certain lectins to bind to the glycoproteins responsible for eliciting mating activity. Electrophoretic analysis of rotifer glycoproteins bound by the lectins ConA, lentil, pea, and fava has identified a small set of 5-7 glycoproteins (Snell & Jarim-Singh, 1992). Since these lectins have the capacity to selectively block mate recognition by binding to these glycoproteins, one of the glycoproteins is almost certainly the mate recognition pheromone (MRP). Work is in progress to purify further and characterize the glycoprotein eliciting male mating. Given the potential biological significance of these glycoproteins, it seemed interesting to ask where they are located on the body surface of females. If localized, their position could provide some indication of their role in signaling males during mating.

The objectives in this work were to use lectins to probe the surface glycoproteins of rotifers in an attempt to localize the MRP. The distribution of the MRP on females may provide insight into its role as a signal for males. Relating MRP distribution to the primary sites of copulation is likely to promote understanding of male copulatory behavior.

Materials and Methods

All experiments utilized the Russian strain of the rotifer *Brachionus plicatilis* O.F. Muller (Snell & Carillo 1984). Standard experimental conditions for mass cultures were 25° C, 15 ppt salinity seawater made with commercial seasalts (Instant Ocean, Aquarium Systems) dissolved in deionized water. Rotifers were fed a diet of *Tetraselmis chuii* and light was provided on a 16:8 L:D photoperiod at a 4000 lux intensity.

Fluorescence time series experiments were performed using 1 to 3 h old female neonates obtained by hatching cysts at 25°C. The neonates were placed in a 24-well tissue culture plate and exposed to 0.1 mg/ml of the lectin Concanavalin A (ConA) conjugated to fluoroisothiocyanate (FITC) in 500 ul of seawater for 0.5, 1, 5, 10, 20, or 30 min. After exposure, females were washed twice in 1 ml of seawater to remove unbound label. Ten to fifteen females were anesthetized with 0.5% tricane methanesulfonate (MS-222), then transferred to a whole mount slide. Rotifers were examined at 250X magnification for fluorescence using a filter block with excitation at 450-490 nm and an emission barrier at 515 nm. Photomicrographs were taken with an automatic exposure system at 1600 or 800 ASA. This procedure was repeated for male neonates which were obtained from ovigerous mictic females isolated from mass cultures.

The intensity of fluorescent labeling was compared for nine lectins conjugated to FITC: *Lens culinaris* (LC) lentil, *Canavalia ensiformis* (ConA) jack bean, *Pisum sativum* (PS) pea, *Vicia faba* (VF) fava bean, *Ulex europaeus* (UE) gorse, *Lycoperison esculentum* (LE) tomato, *Bauhinia purpurea* (BP) camel's foot tree, *Erythrina corallodendron* (EC) coral tree, and *Glycine max* (GM) soybean (Sigma Chemical Company). These lectins were chosen because they represent a variety of oligosaccharide binding specificities. The lectins LC, ConA, VF, and PS bind to terminal α-D-mannosyl and α-D-glucosyl residues. EC, GM and BP bind

to N-acetyl-D-galactosamine and LE binds N-acetyl-β-D-glucosamine oligomers. UE has specificity for L-fucose residues. Female neonates were placed in 0.1 mg lectin-FITC/ml seawater for five min. After exposure, rotifers were washed, anesthetized, and examined for fluorescence.

Fluorescence was quantified using an image analysis system coupled to an Olympus BH-2 microscope. Black and white video images were recorded with a Javelin CCD camera (model JE-7242) at 50X magnification. These images were digitized with a Data Translation QuickCapture card on a MacIntosh ci computer. Image data was processed using NIH Image 1.43 software which permitted measurement of fluorescence intensity on a gray scale ranging from 1-256.

In female lectin blocking experiments, female neonates were placed in 1 ml of 0.1 mg/ml unlabeled ConA, LC, PS, or VF lectins for 30 min. Control females were exposed to seawater without lectins. The animals then were washed in 1 ml of seawater and exposed to 0.1 mg ConA-FITC/ml for 3 min. Animals were washed again in 1 ml of seawater, anesthetized, and photographed as previously described.

Experiments examining the degradation of the mate recognition glycoprotein were performed by transferring 10 ul of seawater containing 15 female neonates into a microcentrifuge tube. Added to this was 18.8 ul of 0.4 M sodium phosphate buffer, pH 8.0 and 1.2 ul (0.6 units) N-glycanase enzyme (genzyme Corp.). N-glycanase hydrolyzes asparagine-linked oligosaccharides from glycoproteins releasing free proteins and oligosaccharides. The reaction was incubated at 37°C for 10 minutes. Control animals were placed in a microcentrifuge tube to which 20 ul phosphate buffer was added, and were incubated along with the experimental group. Following incubation, females were washed twice in 1 ml of seawater and exposed to 0.1 mg ConA-FITC/ml for

3 minutes. Females then were washed again in 1 ml of seawater, anesthetized, and photographed.

Results

Our initial approach to localizing the MRP was to expose males and females to ConA-FITC for various times and determine the time sequence of fluorescent labeling of rotifer structures. The hypothesis was that the structures with the highest affinity for lectin binding will label first and most intensely. The structure first exhibiting fluorescence in both males and females was the corona (Table I). Several exposure times were examined; those exceeding one hour produced excessive fluorescence which obscured structural detail, probably through non-specific binding. The coronas of males and females also fluoresced most intensely, with considerably weaker fluorescence visible in the buccal field and mastax of females and the tip of the penis in males. The tip of the foot and lorica margin in females fluoresced weakly after 30-min exposure. The prostate gland in males fluoresced intensely after 30-sec exposure, but intensity did not increase after 30 min. In contrast to the structures described above, which consistently labeled with the same intensity, fluorescence of lateral and dorsal antennas was often observed, but varied among females.

Photomicrographs of the female time series demonstrate the distribution of fluorescence and its intensity after exposure to ConA-FITC (Fig. 1). The intensity of fluorescence increased markedly as exposure times increased from 30 seconds to five minutes. Exposures of 5 to 20 min produced similar fluorescence intensities, but after 30 min fluorescence intensity was visibly greater. Variation in fluorescence intensity among individuals in the same treatment was very small compared to the differences between treatments. Similar results were obtained from a male time series (data not shown).

Labeling of rotifer females with the nine FITC labeled lectins was compared for pattern and intensity of fluorescence. Rotifers exposed to UE-FITC, LE-FITC, BP-FITC, EC-FITC, and GM-FITC lectins for 30 min yielded no visible fluorescence on any structure. In contrast, labeling female rotifers with the lectins ConA-FITC, PS-FITC, VF-FITC, and LC-FITC produced intense fluorescence. The same pattern of fluorescence was observed for all four lectins, but there were differences in intensity. Image analysis of fluorescence intensity over the body of females revealed marked contrasts (Fig. 2). Fluorescence was highly localized in the coronal region producing a large single peak of fluorescence. Probably the most important feature of this signal is its contrast with the background. Contrast ratios of corona/body fluorescence intensity varied from 2 for LC to 19.5 for VF, with intermediate ratios of 14.6 and 4.6 for PS and ConA, respectively.

Lectin binding to surface glycoproteins on females and males was investigated further to determine which lectin is the best blocker of fluorescent labeling by ConA-FITC. Females were exposed to unlabeled Con A, PS or LC for 30 minutes, followed by a 3 minute exposure to ConA-FITC (Fig. 3A). Fluorescence of controls was substantially greater than that exhibited by females exposed to the lectin blockers. Pre-exposure to ConA effectively blocked most of the ConA-FITC binding sites, whereas pre-exposure to PS and LC only partially blocked these sites. In males, coronal fluorescence also was effectively blocked by ConA and PS binding, and to a lesser extent by LC binding (Fig. 3B). However, fluorescence of the internal prostate gland was unaffected by the lectin blockers.

Selective *in situ* degradation of glycoproteins in males and females was investigated using N-glycanase. The expectation was that N-glycanase deglycosylation of surface glycoproteins would reduce lectin binding and fluorescent labeling. Males and females exposed to N-glycanase for 10 minutes

were compared to controls incubated similarly but without enzyme. Coronal fluorescence in controls greatly exceeded N-glycanase treated females indicating degradation of ConA binding sites (Fig. 4). Males exhibited a similar pattern, but the photomicrographs are not shown.

Discussion

The rapid appearance and strong intensity of fluorescence on the corona of females suggests that this structure has the greatest affinity for the lectin probes and the highest density of binding sites. Strong fluorescence was visible in only 30 sec on the corona as compared to the lorica margin where fluorescence was visible only after 20 minutes. Sharp differences in label intensity over the body surface of females raises the question of what the lectin probes are actually binding to. Our hypothesis is that the lectins bind directly to the mate recognition pheromone (MRP) on females and that the intensity of fluorescence is directly proportional to the abundance of MRP molecules on their body surface. An alternative explanation is that lectins do not bind to the MRP, but to a closely associated glycoprotein. This latter possibility is unlikely because lectin affinity chromatography with LC and ConA was used to isolate biologically active MRP from crude rotifer homogenates (Snell & Jarim-Singh 1992). The purification was successful because LC and ConA bound directly to the MRP rather than other glycoproteins which lack the ability to elicit male mating responses.

Fluorescently labeled lectins allowed us to visualize the location of the surface glycoproteins responsible for mate recognition. Rotifer males presumably also can detect this signal in the form of the MRP. The MRP signal should be easily distinguishable from background judging from the contrast ratios we observed. The uneven distribution of the MRP over the body surface of females is an important observation because it helps explain the copulatory behavior of

males. Males usually first contact females along their lorica margins where they detect a species-specific signal. Males begin circling the female, maintaining coronal contact as they skim over her body surface (Snell & Hawkinson, 1983). After several seconds, males make their way to the coronal region where penetration occurs and sperm transfer takes place. Snell & Hoff (1987) showed that in *B. plicatilis* 85% of all copulations occur through the corona, 11% through the foot opening, and 4% at other sites. By localizing penetration to the coronal region of females, males avoid the lorica which poses more resistance than coronal membranes. This pattern of MRP distribution on the body surface of females appears to act as a beacon guiding males to the most favorable site for sperm transmission. By searching for the most intense pheromonal signal, males are led to the corona where fertilization has the best chance for consummation.

The pattern of lectin binding observed suggests that the MRP is densest in the coronal region where it may be associated with ciliary membranes. Merkel *et al.* (1981) found the ciliary membrane glycoproteins of *Paramecium* to be involved in regulation of mating behavior. Structural analysis of these glycoproteins showed that the two predominant sugars were glucose and mannose. Results from our lectin binding experiments suggest that glucose and mannose also are major components of the carbohydrate moeity that plays a role in mate recognition in *B. plicatilis*. Lectins also have been used successfully in describing the distribution of surface glycoproteins in *Paramecium* and elucidating the structure of their carbohydrate moieties by Allen *et al.* (1988). Conjugation in the ciliate *Tetrahymena pyriformis* is inhibited by ConA, probably through interaction with specific membrane-bound glycoproteins (Ofer et al., 1976; Frisch et al., 1977). In *Tetrahymena*, conjugation inhibition occurs by binding of ConA to receptors localized at the junction between conjugating cells (Frisch & Loyter 1977; Watanabe et al. 1981; Pagliaro & Wolfe 1987). These

authors used a variety of techniques, consistently obtaining results that supported the hypothesis that the ConA receptor and the mating receptor are the same molecule. Pagliaro & Wolfe (1987) showed that conjugation activity is controlled by ConA binding to individual receptors rather than by the cross-linking or reorganization of receptors. Using SDS-PAGE, these authors identified a glycoprotein with molecular weight of 23000 D that was a very good candidate for the mating receptor.

The MRP on the lorica margin may be associated with the cuticle where the presence of glycoproteins has been demonstrated. Koehler (1965) exposed intact females of the rotifer *Asplanchna brightwelli* to a variety of enzymes in experiments to elucidate the biochemical nature of the cuticle. He found with transmission electron microscopy that the protease pronase removed the entire cuticle and that glycohydrolases α–amylase and β–amylase removed parts of the cuticle. These results suggested that the cuticle had glycoprotein components. Brodie's (1970) EM study of the development of the cuticle in *A. brightwelli* demonstrated that cuticle components are secreted from hypodermal bulbs. The cuticle and material in the bulbs stained with ruthenium red indicating that they were composed of glycoprotein.

The binding of different types of lectin probes suggests particular structural characteristics for the MRP. Binding experiments with ConA produced the greatest fluorescence, whereas labeling with LC lectin yielded the least. This suggests that there are fewer LC binding sites. The LC sites are probably a subset of the ConA sites, suggesting that LC perhaps is more selective for the MRP. The experiments using lectin blockers prior to labeling further supports this interpretation. Pre-treatment with ConA was most effective at reducing fluorescent labeling with ConA-FITC. Pre-treatment with LC and PS lectins only partially limited ConA-FITC labeling, suggesting that they bind to a subset of

ConA sites. Another supporting observation is that electrophoretic examination of silver stained proteins from affinity purified MRP samples indicates that ConA binds more glycoproteins than LC (Snell & Jarim-Singh, 1992). EC, GM, LE, UE, and BP lectins failed to bind to the surface glycoproteins of *B. plicatilis*. All of these lectins lack the mannose/glucose affinity of ConA, LC, PS, and VF lectins (Goldstein & Poretz, 1986). These differential patterns of lectin binding can be used to deduce structural features of the carbohydrate moieties of glycoproteins (Osawa & Tsuji, 1987).

Deglycosylation with N-glycanase substantially reduced lectin labeling. This clearly illustrates the importance of the carbohydrate moeity in lectin binding. *In situ* N-glycanase degylcosylation of glycoproteins on the body surface of live females markedly reduced their ability to elicit mating reactions from males (Snell & Nacionales, 1990). These two observations clearly indicate that the same oligosaccharides critical for lectin binding also are essential for mate recognition.

As in females, the coronal region of males labeled rapidly and intensely. This at first seemed to be a peculiar result since our model for mate recognition suggested that females have the MRP, whereas males possess the MRP receptor. The lectin labeling indicated strong binding to regions on males where the MRP receptor was hypothesized to be, but why should lectin probes bind to both signal and receptor? One explanation could be that recognition is based on a homotypic type of carbohydrate-protein interaction (Sharon & Lis, 1989). In this model, both males and females possess carbohydrates and protein receptors which are complimentary. Lectins binding to glycoproteins involved in mate recognition therefore would light up both males and females. Similar results in males and females for the lectin pre-treatment blocking experiments further supports this interpretation. Another supporting observation is that the female

signal and male reception make equal contributions to mating discrimination (Snell & Hawkinson, 1983), a result expected if complimentary recognition molecules are present on both sexes.

In observations of mating behavior we saw numerous male-male encounters, yet none resulted in initiation of mating behavior. Males clearly are capable of discriminating females from other males. The basis for this discrimination may lie in the density of MRP sites on the lorica margin of females. Although the lorica margin labeled weakly in females, it was clearly detectable. This was not the case for males where no fluorescence was visible after 30 minutes of exposure. Perhaps this indicates the absence of a signal on the lorica margin of males, making mating responses unlikely in male-male encounters.

Acknowledgments

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Snell, T. W. 1989. Systematics, reproductive isolation and species boundaries in monogonont rotifers. *Hydrobiologia*, vol. 186/187, pp. 299-310.

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Snell, T. W. & P. D. Morris. 1992. Sexual communication in copepods and rotifers. *Hydrobiologia*, in press:

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Tables

Table I. Time sequence of fluorescent labeling of females and males exposed to ConA-FITC. The X's denote the presence of fluorescence, not its intensity.

Structure	Exposure (min)					
Females	0.5	1	5	10	20	30
corona	X	X	X	X	X	X
buccal field		X	X	X	X	X
mastax			X	X	X	X
tip of foot				X	X	X
lorica margin					X	X
Males						
corona	X	X	X	X	X	X
prostate gland	X	X	X	X	X	X
tip of penis					X	X

Figure Captions

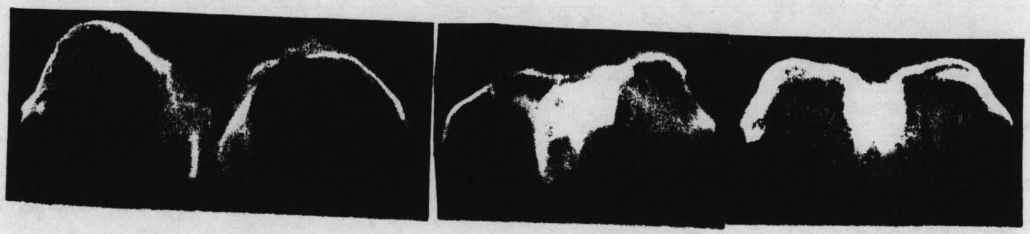
Figure 1. The effect of ConA-FITC labeling on female coronal fluorescence. The micrographs shown are typical of individuals exposed for the indicated times.

Figure 2. Comparison of fluorescent intensity resulting from the binding of FITC labeled lectins to surface glycoproteins. The orientation on the X-axis is a horizontal animal in dorsal view with corona nearest the Y-axis. FU is arbitrary fluorescence units. The lectins are: *Lens* (LC), *Pisum* (PS), ConA, and *Vicia* (VF).

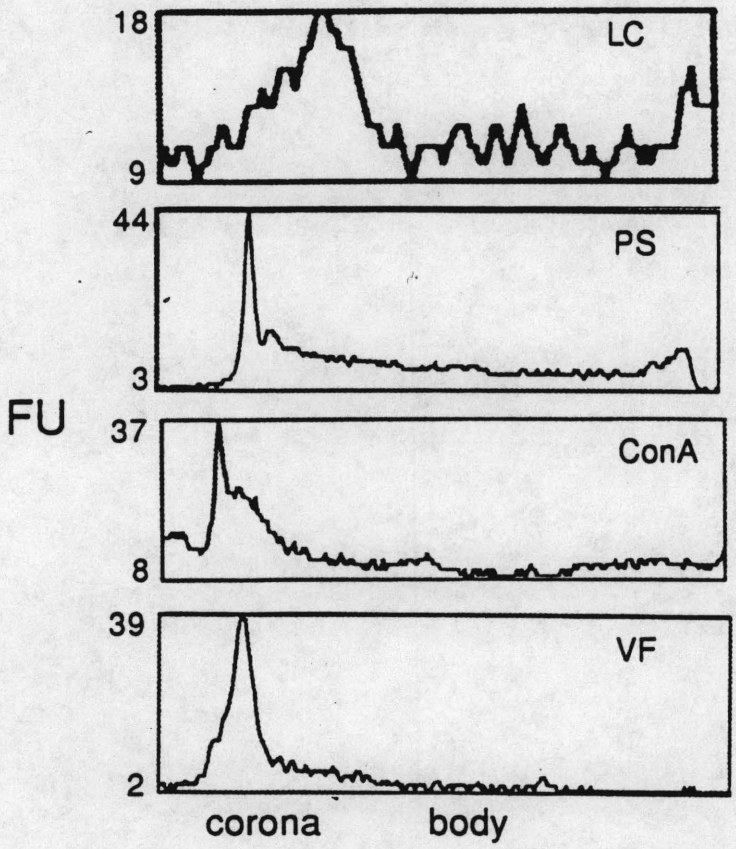
Figure 3. Lectin blocking followed by ConA-FITC labeling of females (A) and males (B).

Figure 4. N-glycanase treated, ConA-FITC labeled females.

0.5 min 1 min 5 min



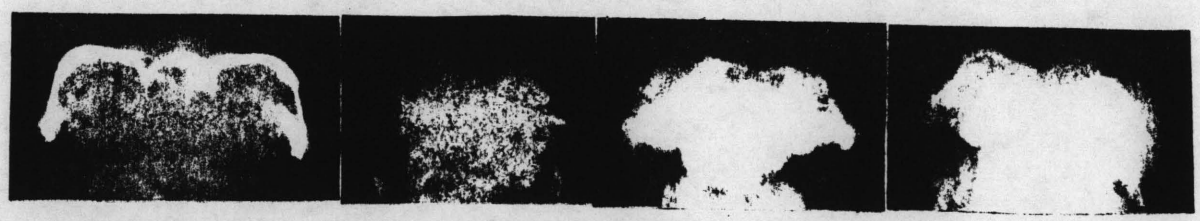
10 min 20 min 30 min


18
9
LC
44
3
PS
FU
37
8
ConA
39
2
VF
corona
body

3

Females

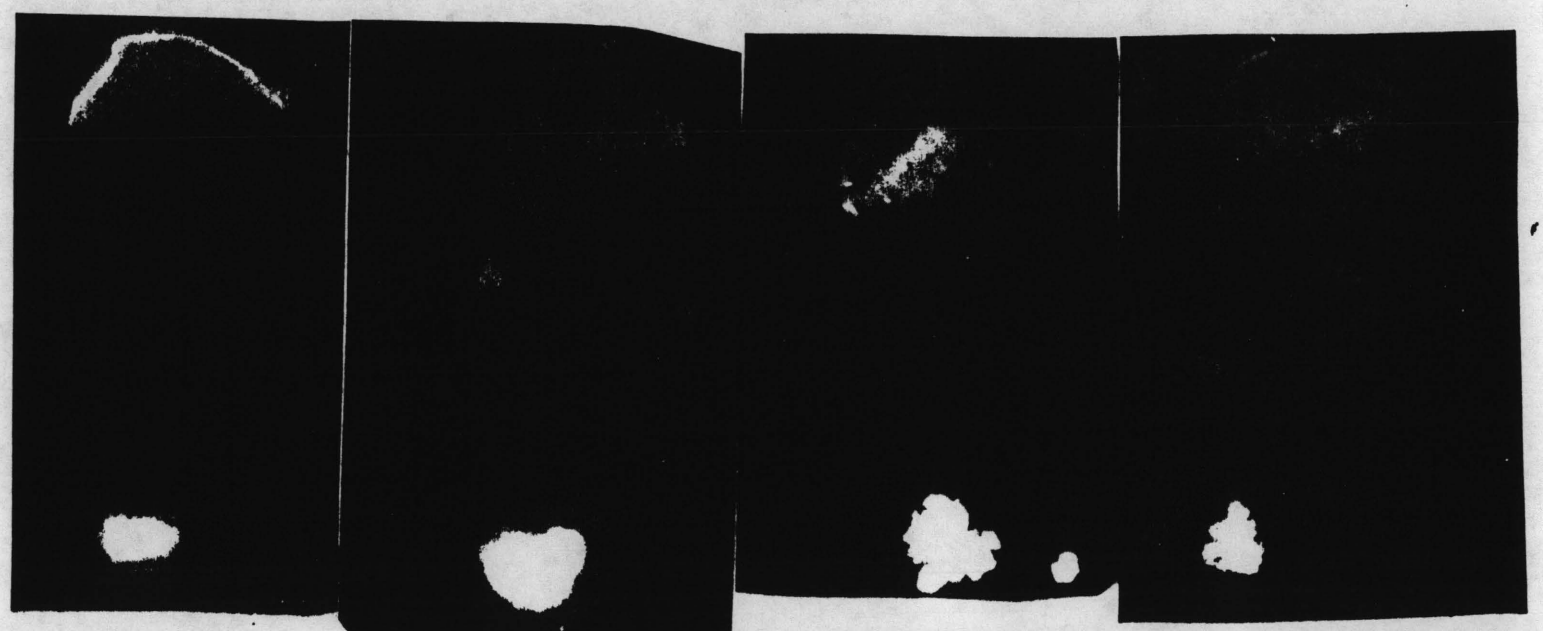
A



Control ConA PS LC

Males

B



Control ConA PS LC

Control N-glycanase

Terry W. Snell
Department of Biology
GA Tech Res Corp - GIT
Atlanta GA 30332-0230

PART I - PROJECT IDENTIFICATION INFORMATION

1. Program Official/Org.	Phillip R. Taylor - OCE
2. Program Name	BIOLOGICAL OCEANOGRAPHY PROGRAM
3. Award Dates (MM/YY)	From: 01/92 To: 06/94
4. Institution and Address	GA Tech Res Corp - GIT Administration Building Atlanta GA 30332
5. Award Number	9115860
6. Project Title	Chemical Communication Among Marine Zooplankton

This Packet Contains
NSF Form 98A
And 1 Return Envelope

SF Grant Conditions (Article 17, GC-1, and Article 9, FDP-11) require submission of a Final Project eport (NSF Form 98A) to the NSF program officer no later than 90 days alter the expiration of the ward. Final Project Reports for expired awards must be received before new awards can be made NSF Grants Policy Manual Section 677).

low, or on a separate page attached to this form, provide a summary of the completed projects and technical information. Be re to include your name and award number on each separate page. See below for more instructions.

PART II - SUMMARY OF COMPLETED PROJECT (for public use)

e summary (about 200 words) must be self-contained and intelligible to a scientifically literate reader. Without restating the oject title, it should begin with a topic sentence stating the project's major thesis. The summary should include, if pertinent the project being described, the following items:

The primary objectives and scope of the project
The techniques or approaches used only to the degree necessary for comprehension
The findings and implications stated as concisely and informatively as possible

PART III - TECHNICAL INFORMATION (for program management use)

references to publications resulting from this award and briefly describe primary data, samples, physical collections, ntions, software, etc. created or gathered in the course of the research and, if appropriate, how they are being made available e research community. Provide the NSF Invention Disclosure number for any invention.

ify to the best of my knowledge (1) the statements herein (excluding scientific hypotheses and scientific opinion) are true and complete, and e text and graphics in this report as well as any accompanying publications or other documents, unless otherwise indicated, are the original of the signatories or of individuals working under their supervision. I understand that willfully making a false statement or concealing a rial fact in this report or any other communication submitted to NSF is a criminal offense (U.S. Code, Title 18, Section 1001).

[redacted]	6-29-94
Principal Investigator/Project Director Signature	**Date**

[redacted]

NSF Form 98A (Rev. 2/92)

PART IV -- FINAL PROJECT REPORT -- SUMMARY DATA ON PROJECT PERSONNEL

(To be submitted to cognizant Program Officer upon completion of project)

The data requested below are important for the development of a statistical profile on the personnel supported by Federal grants. The information on this part is solicited in resonse to Public Law 99-383 and 42 USC 1885C. All information provided will be treated as confidential and will be safeguarded in accordance with the provisions of the Privacy Act of 1974. You should submit a single copy of this part with each final project report. However, submission of the requested information is not mandatory and is not a precondition of future award(s). Check the "Decline to Provide Information" box below if you do not wish to provide the nformation.

Please enter the numbers of individuals supported under this grant.
Do not enter information for individuals working less than 40 hours in any calendar year.

	Senior Staff		Post-Doctorals		Graduate Students		Under-Graduates		Other Participants [1]	
	Male	Fem.	Male	Fem.	Male	Fem.	Male	Fem.	Male	Fem.
A. Total, U.S. Citizens	1					1	1	1		
B. Total, Permanent Residents										
U.S. Citizens or Permanent Residents [2]:										
American Indian or Alaskan Native										
Asian.....							1			
Black, Not of Hispanic Origin.....										
Hispanic										
Pacific Islander										
White, Not of Hispanic Origin	1					1		1		
C. Total, Other Non-U.S. Citizens										
Specify Country 1.										
2.										
3.										
D. Total, All participants (A + B + C)	1					1	1	1		
Disabled [3]										

☐ Decline to Provide Information: Check box if you do not wish to provide this information (you are still required to return this page along with Parts I-III).

[1] Category includes, for example, college and precollege teachers, conference and workshop participants.

[2] Use the category that best describes the ethnic/racial status fo all U.S. Citizens and Non-citizens with Permanent Residency. *(If more than one category applies, use the one category that most closely reflects the person's recognition in the community.)*

[3] A person having a physical or mental impairment that substantially limits one or more major life activities; who has a record of such impairment; or who is regarded as having such impairment. *(Disabled individuals also should be counted under the appropriate ethnic/racial group unless they are classified as "Other Non-U.S. Citizens.")*

AMERICAN INDIAN OR ALASKAN NATIVE: A person having origins in any of the original peoples of North America and who maintains cultural identification through tribal affiliation or community recognition.

ASIAN: A person having origins in any of the original peoples of East Asia, Southeast Asia or the Indian subcontinent. This area includes, for example, China, India, Indonesia, Japan, Korea and Vietnam.

BLACK, NOT OF HISPANIC ORIGIN: A person having origins in any of the black racial groups of Africa.

HISPANIC: A person of Mexican, Puerto Rican, Cuban, Central or South American or other Spanish culture or origin, regardless of race.

PACIFIC ISLANDER: A person having origins in any of the original peoples of Hawaii; the U.S. Pacific territories of Guam, American Samoa, and the Northern Marinas; the U.S. Trust Territory of Palau; the islands of Micronesia and Melanesia; or the Philippines.

WHITE, NOT OF HISPANIC ORIGIN: A person having origins in any of the original peoples of Europe, North Africa, or the Middle East.

Part II - Summary of Completed Project

A central role for sex pheromones in communication among marine animals has long been postulated. Chemical communication is thought to be involved in many critical life cycle processes like locating conspecifics, recognizing them as potential mates, and transferring sperm. Despite the evolutionary significance of these signals, little is known about the type of molecules employed and the basis for discrimination. For common groups like copepod and rotifer zooplankton, behavioral evidence has suggested the existence of sex pheromones, but none has been isolated and characterized. This is in contrast to terrestrial environments where insect sex pheromones have been well characterized and the molecular basis for their action is well understood for several groups. The main objective of this project was to identify the chemical responsible for mate recognition in the marine rotifer *Brachionus plicatilis*, to examine its role in the maintenance of reproductive isolation, and to use these techniques to examine the role of surface glycoproteins in regulating marine copepod mating. We successfully isolated a glycoprotein pheromone responsible for mate recogntion in rotifers, confirmed its biological activity in receptor binding assays, and prepared an antibody against it that blocked mate recognition by males. The presence of surface glycoproteins on several species of copepods was demonstrated at sites thought to be important in mating.

Part III

List of Publications

Snell, T.W. and P.D. Morris. 1993. Sexual communication in copepods and rotifers. Hydrobiologia 255/256:109-116.

Snell, T.W., P.D. Morris, & G.A. Cecchine. 1993. Localization of the mate recognition pheromone in the marine rotifer *Brachionus plicatilis*. J. Exp. Mar. Biol. Ecol.165:225-235.

Snell, T.W. and M.J. Carmona. 1994. Surface glycoproteins in copepods: potential signals for mate recogniton. Hydrobiologia, in press.

Snell, T.W. and B.D. Moffat. 1994. *In vivo* fluorescent biomarkers of toxicity in the rotifer *Brachnious plicatilis*. Ecotoxicol. Environ. Safety, in press.

Snell, T.W. and M.J. Carmona. 1994. Comparative toxicant sensitivity of sexual and asexual reproduction in the rotifer *Brachionus calyciflorus*. Envir. Toxicol. & Chem., in press.

Snell, T. W., Rico-Martinez, R., Steadman, L. N., and Battle, T.E. 1994. Identification of a sex pheromone from a rotifer. Marine Biology, submitted.

Dusenbery, D.D. and Snell, T.W. 1994. Diffusible pheromones are of no use to small organisms. J. Chemical Ecology, submitted.

Rico-Martinez, R. and Snell, T.W. 1994. Copulatory behavior and pheromone blocking of male receptors in *Brachionus plicatilis* (Muller) Rotifera. Hydrobiologia, submitted.

Relevant Presentations:

American Society of Limnology & Oceanography, Feb 1992, Santa Fe, New Mexico "Biochemical characteristics of the mate recognition pheromone in the marine rotifer *Brachionus plicatilis*". T.W. Snell and M. Jarim

Fifth International Copepod Symposium, June 1993, Baltimore, Maryland "Surface glycoproteins in copepods: possible signals for mate recognition" T.W. Snell and M.J. Carmona

American Society of Limnology & Oceanography, Feb 1994, San Diego, California "Lectins bind to surface glycoproteins on copepods and inhibit mate guarding" T.W. Snell and D.J. Lonsdale

Seventh International Rotifer Symposium, June 1994, Mikolajki, Poland "Copulatory behavior and pheromone blocking of male receptors in *Brachionus plicatilis* (Rotifera)", R. Rico-Martinez and T.W. Snell

Description of Data Acquired

A) Isolation and Characterization of the Mate Recognition Pheromone in the Marine Rotifer *Brachionus plicatilis*

Glycoprotein Purification - Treatment of females with N-glycanase, a glycohydrolase that releases N-linked oligosaccharides from glycoproteins, significantly reduced the probabilty of male copulation (Snell and Nacionales 1990). Treatment of females with several mannose/glucose binding lectins also reduced the number of copulation attempts by males. Using lectins labeled with FITC, the highest concentration of the putative mate recognition glycoprotein was localized in the corona of females (Snell et al. 1993). Sequential lectin affinity chromatography beginning with *Lens culinaris* retained a small subset of proteins from a crude homogenate of rotifer biomass (Snell et al. 1994). *Lens* lectin retained eight to ten proteins in sufficient quantities to be visualized on SDS-PAGE. The second step in the purification utilized the lectin *Tetragonolobus purpureas* which has fucose binding affinity. Passage of the sample over this column yielded a single, apparently homogenous protein, gp29.

Biological Activity of gp29 - Newborn males were exposed to a purified sample of gp29 at a concentration of 170 μg/mL. The expectation was that if gp29 were the MRP, it would bind to the male receptors and reduce the ability of males to recognize females. The number of male-female encounters and attempted copulations were compared in glycoprotein treated and control males. Compared to control males exposed only to buffer, the gp29 treated males attempted 93% fewer copulations (χ^2=26.7, P<.001). Biotinylation permitted the localization of gp29 binding on male rotifers using an avidin fluorescent label. Males were exposed to the biotinylated gp29 for 30 minutes, washed, and then exposed to fluorescein labeled beads cross-linked to avidin. No fluorescence was visible in the control, but a sharp band of fluorescence in the gp29 treated male indicated localized binding in the anterior ciliary band called the corona.

Antibody to gp29 - A polyclonal antibody against gp29 was raised in rabbits. This antibody recognizes only a single protein in crude homogenates of rotifer biomass (Figure 1). The anti-gp29 bound to the surface of females, reducing the number of copulation attempts by males. Females treated with 110 µg of IgG containing anti-gp29 elicited only 14% and 12% of the male copulation attempts as the pre-immunization and buffer controls, respectively (χ^2=70.4, P<.001). Localization of anti-gp29 binding sites on female rotifers was accomplished by biotinylating post-immune serum. Since anti-gp29 was among the IgG fraction of this serum, the biotinylated antibody bound to gp29 molecules on the body surface of females. Unbound antibody was removed by washing, then avidin-fluorescein beads were added. Strong fluorescent labeling localized in the corona.

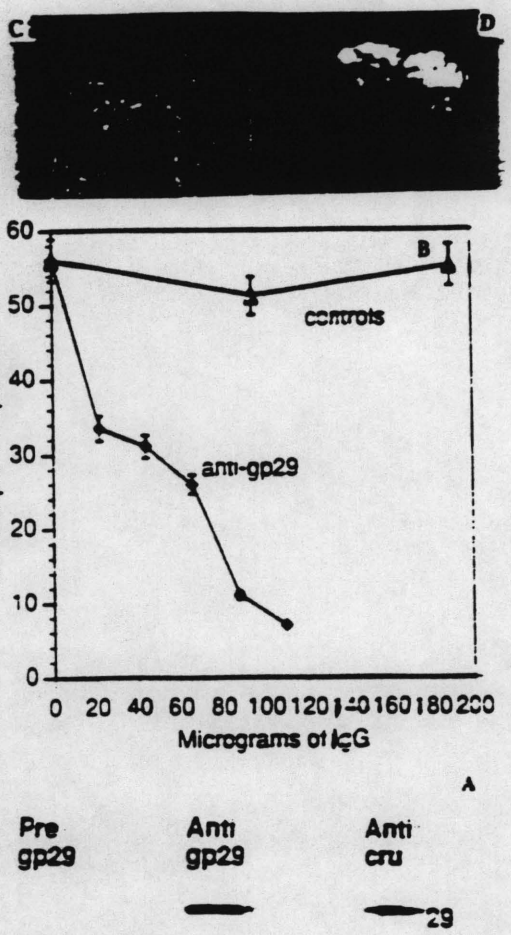


Figure 1. Characterization of antibodies to gp29. (A) Pre gp29 and anti gp29 samples are western blots of lectin affinity purified gp29. Anti cru sample is crude rotifer homogenate. (B) Controls were treated with purified IgGs from pre-immune serum. Anti-gp29 was treated with purified IgGs from post-immune serum. (C) Control female was exposed to biotinylated pre-immune serum, then to avidin-fluorescein beads. (D) female was treated similarly, but exposed to biotinylated post-immune serum.

Attachment of gp29 to beads - To determine whether gp29 alone is sufficient to elicit male mating behavior, we attached gp29 to 60 µm diameter sepharose beads. Freeze-killed females were used as positive controls and males attempted copulation in 54% of encounters. When surface glycoproteins on these females were deglycosylated with N-glycanase, males attempted copulation in only 12% of encounters. Beads with glycyl-leucine attached served as negative controls and elicited no male responses in 200 encounters. Beads with gp29 attached elicited male copulation attempts in 38% of encounters which is 2/3 of their response to the freeze-killed females. Deglycosylation of the gp29 beads resulted in males attempting copulation in only 2.3% of encounters.

B) Variation in the Mate Recognition Pheromone among *Brachionus plicatilis* strains and Other Rotifer Species

Reactivity of gp29 antibody from the Russian strain was tested towards other strains (Rico-Martinez and Snell 1994). Six strains were examined including 3 L types (RUS-Russian, AUS-Austrian, GP-Colorado) and 3 S types (HAW-Hawaiian, KOS-Koshiki, Japan, S1-Spain). The most anti-gp29 binding was to the Russian and Colorado females (Figure 2). The Austrian and Hawaiian females had about 1/2 the binding and Koshiki and Spanish females had about 1/7 of the Russian and Colorado females. These results clearly demonstrate the differential binding of anti-gp29 to *B. plicatilis* strains and suggests that the structure of gp29 may differ among strains.

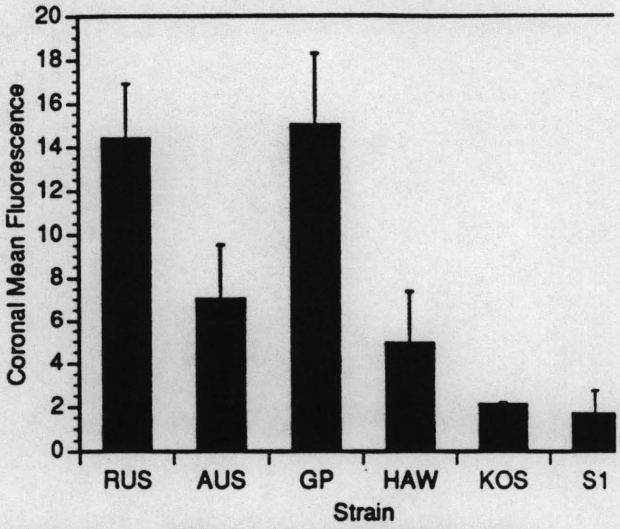


Figure 2. Male receptor binding assay. Coronal fluorescence measures the amount of anti-gp29 binding to males. Vertical lines indicate standard error.

We are continuing to probe the limits of anti-gp29 cross-reactivity to other brachionid species and to other rotifer families.

C) Surface Glycoproteins as Signals in Copepod Mating

The mechanism male copepods use to recognize mates is not well understood. Both chemical and mechanical cues have been implicated, but the relative importance of these is not known. We have developed techniques for selectively probing surface glycoproteins in copepods using fluorescently labeled lectins (Snell and Carmona 1994) to develop insight into their role in mate recognition. Calanoids *Labidocera aestiva*, *Centropages hamatus*, and *Acartia tonsa* were collected in the northern Gulf of Mexico and screened with 12 lectins representing a wide variety of carbohydrate affinities. The harpacticoid *Coullana canadensis* originally collected from Florida was screened for the same 12 lectins. The freshwater calanoid *Skistodiaptomus pygmaeus* and the cyclopoid *Mesocyclops edax* also were investigated and compared to the marine species. The sites of lectin binding were consistent across species. Most fluorescence was observed in the urosome at the caudal rami, gonopore, margin of the genital segment, and urosome segment junctions. The signal contrast (signal/background ratio) along the urosome ranged from 3-51 which seems ample for males to discriminate a glycoprotein signal from noise. Our observations clearly demonstrate the presence of glycoproteins at sites expected to be important in copepod mate recognition.